

Induction of Metamorphosis in the Sea Urchin, *Pseudocentrotus depressus*, using L-Glutamine

IKUKO YAZAKI and HIROKI HARASHIMA

Department of Biology, Faculty of Science, Tokyo Metropolitan University
Minamiohsawa 1-1, Hachiohji Tokyo 192-03, Japan

ABSTRACT—Larvae of the sea urchin, *Pseudocentrotus depressus*, were induced to metamorphose using 10^{-6} – 10^{-4} M L-glutamine. After being subjected to 6×10^{-5} M glutamine, the larvae ceased swimming, began to retract their arms, extruded primary podia through an opening of the larva at a vestibule of echinus rudiment (ER) and then everted the ER, requiring 1–9 hr, 7–11 hr, 7–38 hr and 12–48 hr (from the time of the recognition of the first larva to the time of the maximum larvae changes), respectively. The effective times needed in the glutamine-treatment were 2 hr for induction of the cessation of larval swimming and the start of arm-retraction, and 4–8 hr to induce the ER-eversion. There was no correlation between the extruding of primary podia and the amount of time needed for the glutamine-treatment. From the difference seen in the effective time of glutamine-treatment, the early changes in metamorphosis; cessation of swimming and start of arm-retraction, are not autonomously linked to the later change; ER-eversion. L-glutamic acid and γ -aminobutyric acid (GABA) did not induce metamorphosis. 10^{-7} M GABA provoked a rapid cramp in the arms and a spreading of spines, and the larva seemed to undergo ER-eversion, but within a few hours they returned to their original larval shape. Thus eversion-like changes induced by GABA may not reach a true ER-eversion due to an absence of process or processes which were induced by the 4–8 hr treatment of glutamine over a long period of time (12–48 hr).

INTRODUCTION

Larvae of marine invertebrates undergo metamorphosis in response to environmental cues [6]. Sands and sediments in the adult habitat cause metamorphosis in larvae of the sand dollar [8] and *Nassarium* [18]. Bacteria and algae substrata are also effective for inducing metamorphosis. In aplysiids and spirorbis (serpulidae), the larvae of each aplysioid or spirorbis species selects a substratum containing their favorite algae in which to settle [7, 20]. In echinoderm, bacterial and algal films, which are prepared by soaking the glass or plastic plates in natural sea water for several days, are effective in inducing metamorphosis of sea star larvae [12, 19] and sea urchin larvae [9, 15, 23]. However, the active factor of these natural cues has been rarely identified. For example, free fatty acid components were extracted from red algae as a chemical inducer of larval settlement and metamorphosis of the sea urchin, *Pseudocentrotus depressus* and *Anthocidaris crassispina* [11], and a pheromonal substance, 980 Da-peptide, was isolated as an active factor in the metamorphosis in the sands in the adult habitat of *Dendraster excentricus* [3].

γ -Aminobutyric acid (GABA), which is known as a neurotransmitter in many species, induced abalone larvae to settle and to undergo metamorphosis [13]. In sea urchins, Burke [2] proposed a neural control of metamorphosis according to his electrical and obsolatory experiments that the larvae of *D. excentricus* were induced to metamorphose by electrical stimulation of their two nerve centers, apical neuropile and oral ganglions, although he noted that GABA

was ineffective [2]. In *Strongylocentrotus intermedius* and *S. milabiris*, L-glutamine, which is known as a precursor of glutamic acid and GABA in the central nervous system of mammals, induced metamorphosis in 100% and 50% of the larvae, respectively [16].

In the present study, the effects of L-glutamine on the metamorphosis of *P. depressus* larvae were examined, and the mechanisms of the glutamine-inducing metamorphosis are discussed.

MATERIALS AND METHODS

Larvae which derived from one pair of a male and a female of *Pseudocentrotus depressus* were reared by the method of Noguchi [17] with slight modifications. The cultures in five 3–l beakers were maintained at $19^{\circ} \pm 2^{\circ}\text{C}$ which were stirred with a paddle attached to a 60 rpm motor. At the onset of feeding (3 days after fertilization), there were 25,000 plutei in 2.5 litres of paper-filtered sea water. Two or three ml of *Chaetoceros gracilis* ($3\text{--}5 \times 10^6/\text{ml}$) were added to each culture every 1 or 2 days [10], and two-thirds of the culture water was changed twice a week. The larval density was initially 10/ml but was diluted to 1/ml at the stage of an 8-armed pluteus. Various developmental stages of larvae were found in culture beakers. Competent larvae to metamorphosis were obtained over a period of one month, beginning from the 40th day after insemination.

Larvae with fully developed ER were selected and transferred into 0.22 μm Millipore-filtered seawater (MFSW) in a 100-ml beaker. To remove microorganisms taken into the larvae as food, the larvae were then kept for 1 day in MFSW. After rinsing twice in MFSW, every 5–10 larvae were randomly apportioned into each well of 12-well plastic plates, which were filled with either 2.5 ml MFSW (control) or with the same volume of various concentrations of L-glutamine- or other substances dissolved in MFSW (adjusted with NaOH at pH 7.8–8.0). Larvae in the wells were retransferred to a well filled with the same medium to avoid diluting the experimental

medium. Usually, L-glutamine treatment did not exceed 24 hr. If the observations were prolonged, the culture medium (MFSW) was changed every 2 days.

RESULTS

Effective concentrations of L-glutamine needed to induce metamorphosis

Metamorphosis of the echinopluteus occurs after settlement, and includes an eversion of the adult rudiment (echinus rudiment: ER) which develop within the larva, resorption of the larval body into the juvenile, and the subsequent differentiation of the adult form [5]. Larvae used for the present experiments were swimming and had the fully grown adult rudiment whose primary podia were moving within the larva but not extruding out the larval body (Fig. 1A). When the spines and the primary podia of ER were exposed outside the larva by eversion of the ER, the larva was considered to be metamorphosed (Fig. 1B). Larval arms began to retract prior to the eversion of the ER, but had not completely retracted by the time of ER eversion. Parts of the larval arms and epaulets were retained on the newly metamorphosed juvenile (Fig. 1B), and remained for an additional 1 or 2 days after eversion of the ER.

Larvae were treated with various concentrations of L-glutamine solutions from 3×10^{-4} to 6×10^{-6} M for 24 hr, washed with MFSW and then maintained in MFSW (Fig. 2). Out of ten larvae in each concentration, larvae rarely metamorphosed after just 24 hr (the end of glutamine-treatment). Most larvae which were treated with glutamine at concentrations of 3×10^{-4} to 3×10^{-5} M were found to metamorphose after 48 hr. At a concentration of 6×10^{-6} M L-glutamine, no metamorphosis had occurred by 48 hr, and only one larva had metamorphosed at 72 hr (Fig. 2). From these results, 6×10^{-5} M L-glutamine was used as a conventional concentra-

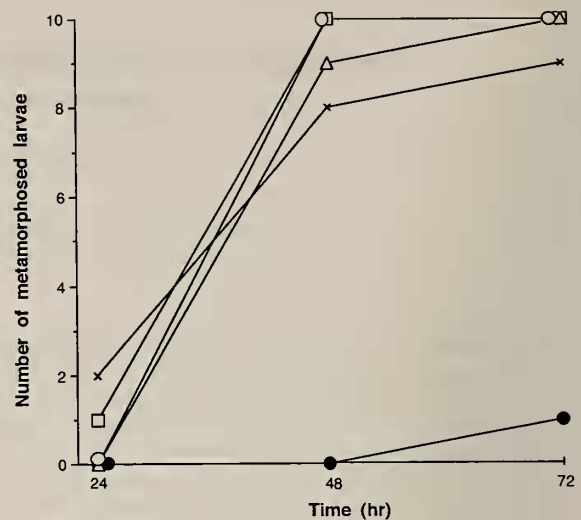


FIG. 2. Effects of L-glutamine at various concentrations. Groups of ten larvae were treated with L-glutamine in MFSW for 24 hr with concentrations of either 6×10^{-6} M (●), 3×10^{-5} M (△), 6×10^{-5} M (×), 1.2×10^{-4} M (□) or 3×10^{-4} M (○). At the beginning of the experiments, all larvae had not metamorphosed. 1.2×10^{-4} and 3×10^{-4} M glutamine induced metamorphosis in all ten larvae after 48 hr. 3×10^{-5} and 6×10^{-5} M glutamine induced metamorphosis in 8 and 9 larvae, but 6×10^{-6} M glutamine did not induce any metamorphosis after 48 hr, with only one larva undergoing metamorphosis after 72 hr.

tion for induction of metamorphosis of *P. depressus* with the resulting metamorphosis usually observed after 48 hr in the following experiments.

Changes in larvae induced using L-glutamine treatment

To analyse the process of metamorphosis, the behaviour and form of larvae were repeatedly checked from the beginning of the glutamine treatment (Fig. 3). Fifty-one larvae were transferred into five wells filled with 6×10^{-5} M gluta-

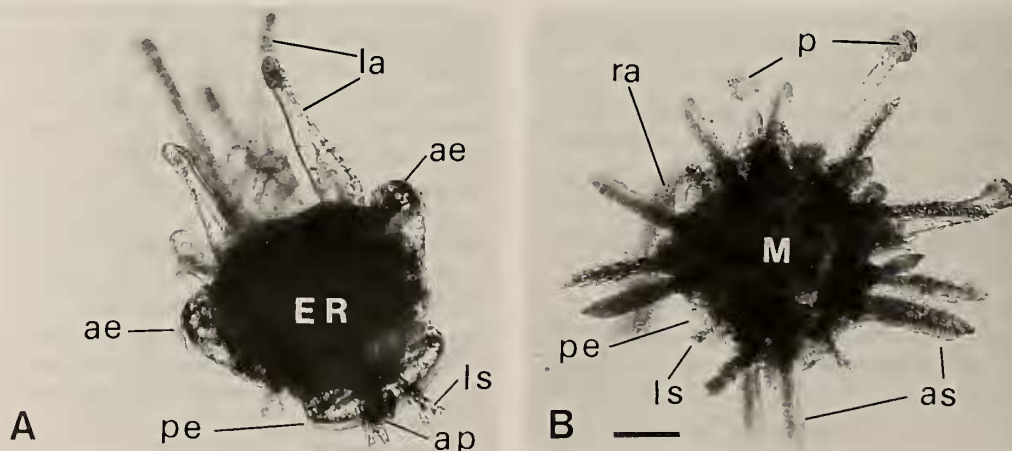


FIG. 1. A competent larva and a juvenile of *P. depressus* which was induced to metamorphose using L-glutamine. A: An 8-armed pluteus larva competent to metamorphose. The larva is swimming around. Neither the spines nor the tube feet (the primary podia) of the echinus rudiment were extruded. B: A juvenile which was metamorphosed after treatment with 6×10^{-5} M L-glutamine for 24 hr, and photographed from beneath with an inverted microscope. la, larval arms; ae, anterior epaulets; pe, posterior epaulets; ap, apical pedicellaria; as, adult spine; ls, larval spine; p, primary podia; ra, retracting arm (out of focus); ER, echinus rudiment; M, mouth. Bar = 100 μ m.

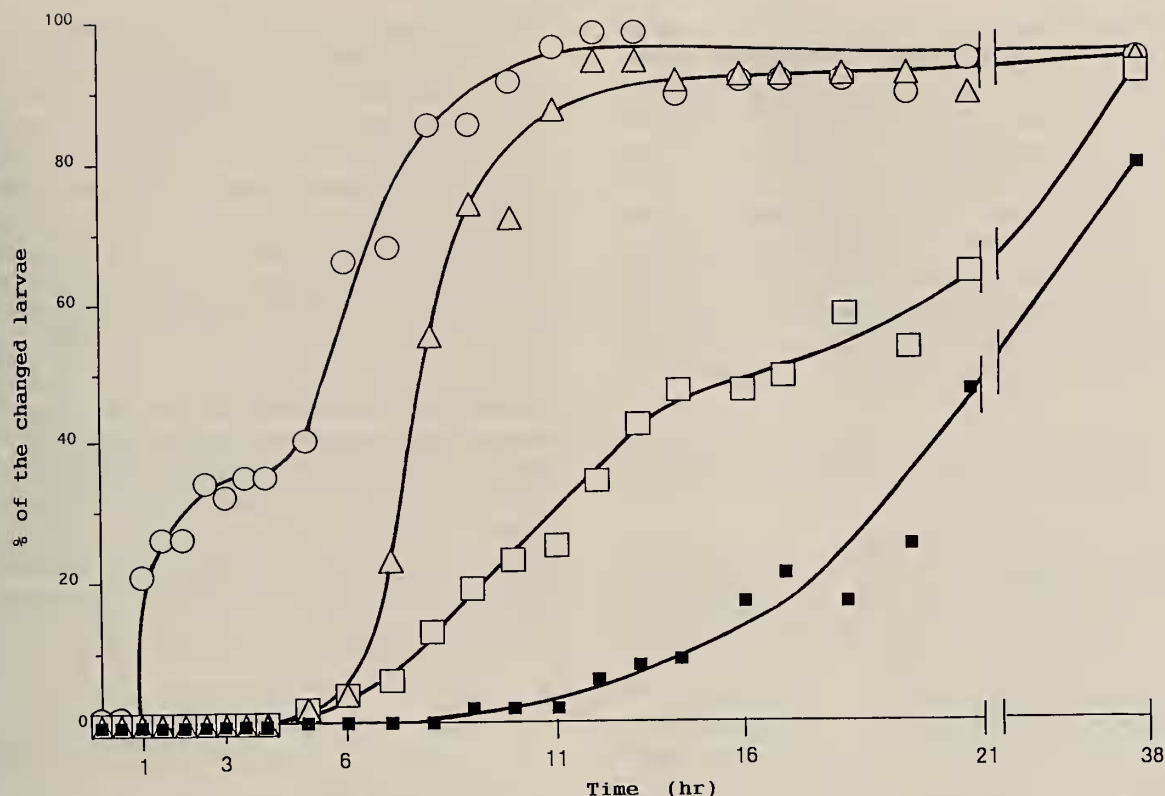


FIG. 3. Time course of larval changes induced by treatment with L-glutamine. Fifty-one competent larvae were kept in 6×10^{-5} M L-glutamine containing MFSW for 21 hr and then transferred into MFSW. Observations were carried out repeatedly on four changes which occurred in each larvae; 1) cessation of swimming ($\circ$), 2) retraction of larval arms (Δ), 3) extrusion of primary podia through an opening of larva at the vestibule of ER ($\square$), and 4) eversion of ER ($\blacksquare$). The echinus rudiments began to evert since each larva ceased swimming and the retraction of arms had occurred. The primary podia seem to be extruded several hours before the ER-eversion.

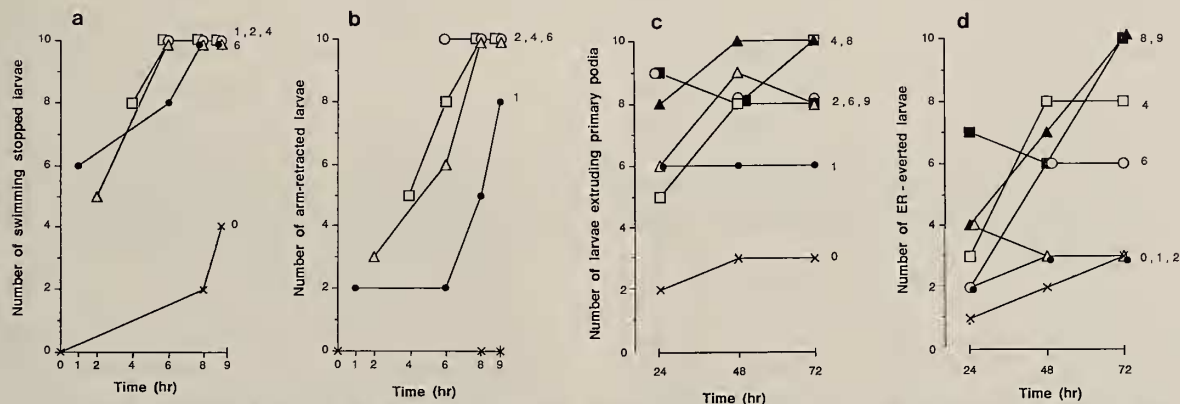


FIG. 4. Larval changes induced by various durations of treatment with L-glutamine. Six 10-larvae groups (10 larvae/well) were treated with 10^{-5} M L-glutamine in MFSW and then transferred into glutamine-free MFSW after 1 hr ($\bullet$), 2 hr (Δ), 4 hr ($\square$), 6 hr ($\circ$), 8 hr ($\blacktriangle$), and 9 hr ($\blacksquare$). Control larvae ($\times$) were reared in glutamine-free MFSW. The larval changes of each group were followed for up to 72 hr and are shown in a) cessation of swimming, b) retraction of arms, c) extrusion of primary podia and d) eversion of the echinus rudiment (ER). a) and b) are shown from zero to nine hours, and c) and d) are from 24 to 72 hr. Numerals to the right of figures indicate the elapsed time of treatment with glutamine. Cessation of larval swimming (a) and the start of arm retraction (b) were induced by 2-hr treatment, although arm retraction progressed with longer durations of treatment. Extrusion of the primary podia (c) seemed to be accelerated by treatment with L-glutamine relative to the controls, but does not have any correlation with the treatment time. To evert the ER (d), larvae must be treated with glutamine for 4–8 hr.

mine-MFSW and kept there for 21 hr. All of the larvae examined were swimming around after the first 30 min. After one hour, 20% of the larvae had stopped swimming and almost all larvae became immobile between 9–10 hr after immersion in glutamine-MFSW. The number of larvae which retracted larval arms and extruded the primary podia began to increase after 7 hr. The arm-retractions were observed in all larvae after 11 hr, while the larvae extruding the primary podia slowly increased. Eversions of ER began after 12 hr. By the end of the treatment (21 hr), ER everted in 50% of larvae examined, and after 38 hr (17 hr after the 21 hr-glutamine treatment), the ER had everted in 88% of the larvae.

Times required for induction of metamorphosis using L-glutamine

Seven 10-larvae groups (10 larvae/well) were prepared. Six groups were placed in wells with 6×10^{-5} M glutamine-MFSW while one group was maintained in MFSW alone as a control. Each group of larvae in glutamine-MFSW was transferred into MFSW at an interval of 1 or 2 hr from the beginning up to 9 hours later (Fig. 4). The movement of larval swimming was affected by very short exposure to glutamine. After a 6-hr treatment, all larvae had ceased swimming (Fig. 4a). Only a 2-hr glutamine treatment made all the larvae completely stop swimming after 6 hr. The effect of a 1-hr treatment was a little unstable (Fig. 4a).

Arms had also begun to retract in all larvae examined after a 6-hr treatment. The start of arm-retraction was much more delayed when the duration of glutamine treatment was shorter, and the progression of arm-retraction was proportional to the duration of glutamine treatment (Fig. 4b). One-hour treatment could induce the start of arm-retraction in all larvae after 24 hr (data not shown), and the arms remained a little shortened.

As the extrusion of the primary podia and the ER eversion were not recognized up to 9 hours, the results achieved after 24 hr are given in Figs. 4c and 4d. The extrusion of podia was also accelerated by glutamine treatment. At 24 hr, in each of the experimental groups (even in the larval group given only a 1-hr treatment), more podia were extruded than in the control group (Fig. 4c). However, there seemed to be no close relationship between the number of larvae whose primary podia had extruded and the duration of glutamine treatment (Fig. 4c).

Eversion of ER at a greater rate than controls could not be induced by either a 1- or 2-hr glutamine treatment. At 24 hr, out of 10 larvae which were treated with glutamine for 9 hr, seven had everted ERs, but in the larvae treated for 8 hr or less, the number of ER everted remained low. At 48 hr, larvae treated for more than 4 hr induced ER-eversion in over 60%, and at 72 hr, 100% ER eversion was recorded in larvae given 8 or 9 hr of treatment (Fig. 4d).

Effects of L-glutamic acid and GABA on metamorphosis

L-glutamic acid, which was deaminated from L-glutamine, was examined at the same concentration (6×10^{-5} M) as L-glutamine for 18–22 hr (Fig. 5). In experiments involving four trials, 69 larvae were treated with glutamic acid, 108 with glutamine and 78 with neither (controls). Observations were carried out for 7 days, although on the 6th and 7th days about half the larvae examined were observed as follows. Among the glutamic acid-treated larvae, only one larva was found to have metamorphosed by the 4th day, after which no more larvae underwent metamorphosis. By contrast, most of the 108 larvae treated with glutamine had metamorphosed by the 2nd day (Fig. 5). Seven larvae, which had metamorphosed by the 4th day, had retracted almost all of their arms to look like black balls and all died. Glutamic acid induced the arm-retractions in 80% of larvae within 2 days (Fig. 6b), but their arms were shortened only slightly. Glutamic acid did not interfere with the larval movement of swimming (Fig. 6a) and the extruding of the primary podia (Fig. 6c).

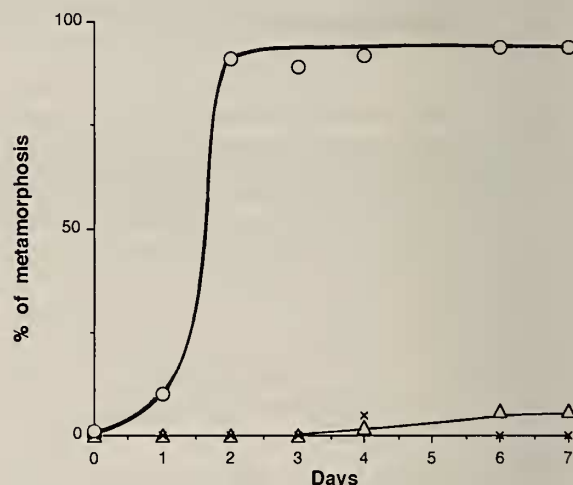


FIG. 5. Effect of L-glutamic acid on metamorphosis compared with that of L-glutamine. Four experiments with different treatment times (18 hr, 20 hr and two experiments with 22-hr treatment) are combined. Observations were continued for 4 days (18-, 20- and 22-hr treatments) and for 7 days (22-hr treatment). The number of experiments totalled 67 for glutamic acid (Δ), 108 for glutamine ($\circ$) and 78 larvae for MFSW (controls) ($\times$). In contrast to the high percentage of metamorphosis-induction using glutamine, glutamic acid induce almost no metamorphosis (there was only one metamorphosed larva among those treated with glutamic acid after 4 days, after which no further metamorphosis occurred).

GABA (γ -aminobutyric acid), a substance which is formed by the decarboxylation of glutamic acid, was applied on each group of 10 competent larvae in concentrations of 5×10^{-5} and 1×10^{-5} M for 24 hr (Exp. 1 in Table 1) or for 8.5 hr (Exp. 2), and at lower concentrations of 1×10^{-6} M or 1×10^{-7} M for 18 hr (Exp. 3) compared with glutamine. When larvae were treated with higher concentrations of GABA for

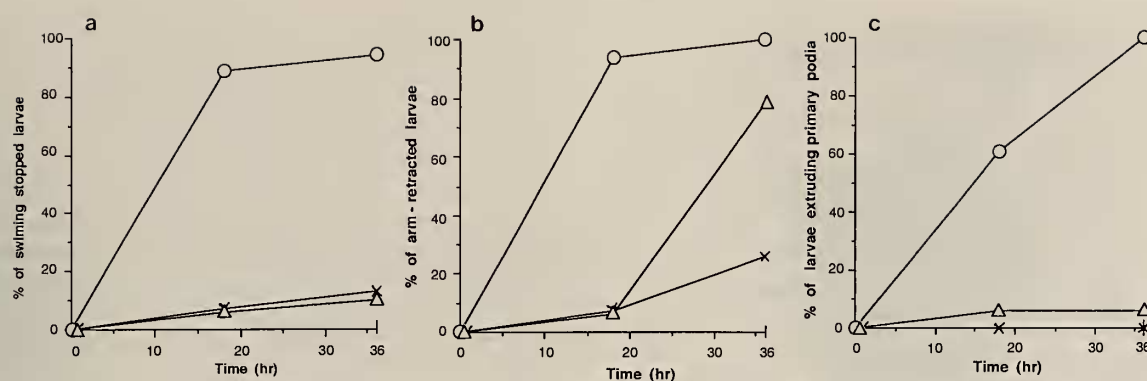


FIG. 6. Changes in larvae treated with L-glutamic acid. Eighteen larvae were treated with 6×10^{-5} M L-glutamic acid (Δ) for 18 hr. To confirm the larval competency to metamorphose, the same concentration of L-glutamine ($\circ$) was applied for the same duration. 16 control larvae ($\times$) were kept in MFSW. L-glutamic acid did not effect cessation of swimming and the extrusion of primary podia, although a slight retraction of arms was observed in 80% of the larvae after 36 hr.

TABLE 1. Effect of various concentration of GABA and various durations of treatment with GABA

Every 10-larvae treated with	Concentrations (M)	Number of metamorphosed larvae		
		at 24 hr	48 hr	72 hr
1) 24-hr treatment				
None	—	0	0	—
L-glutamine	3×10^{-4}	7	8	—
	6×10^{-5}	1	0	—
GABA	5×10^{-5}	5	4*	—
	1×10^{-5}	6	5*	—
2) 8.5-hr treatment				
None	—	0	0	1
L-glutamine	3×10^{-4}	6	10	10
	6×10^{-5}	3	8	9
GABA	5×10^{-5}	0	0	2
	1×10^{-5}	1	1	2
3) 18-hr treatment				
None	—	0	0	0
L-glutamine	6×10^{-6}	1	6	7
GABA	1×10^{-6}	1	3	0
	1×10^{-7}	1	0	0

All larvae had not begun metamorphosis at the start of glutamine- or GABA-treatment. * The echinus rudiments (ER) were exposed but the larvae was dead. In the Exp. 1), the number of metamorphosed larvae (—) were not counted after 72 hr.

24 hr, about half the larvae exposed their spines as the echinus rudiments everted, and they had all died after 48 hr (Exp. 1 in Table 1). As shown in Exp. 2 in Table 1, after reducing the duration of treatment from 24 hr to 8.5 hr, most of the larvae treated with GABA had not exposed their spines at 48 hr, although at 72 hr, 20% had exposed their adult spines at either concentration of GABA. These ER-everted-like larvae seemed to be alive because ciliary movements at the epaulets were observable, but they did not extend any primary podia, and most of their larval arms had not been retracted (Fig. 7). When the concentrations of GABA were reduced to 10^{-6} M or 10^{-7} M, spines of ER

spread out and the ER seemed to be everted in 10% (10^{-7} M) at 24 hr and 30% (10^{-6} M) at 48 hr (Exp. 3 in Table 1). However, these spread spines closed again by the next day. In each of the experimental series, a high ratio of glutamine-treated larvae metamorphosed whereas almost none of the larvae cultured in MFSW (non-treated larvae) metamorphosed.

Figure 8 shows the larval changes in the extrusion of the primary podia and the eversion of ER in Exp. 3 presented in Table 1. Fifteen min after being transferred into 10^{-7} M GABA, 80% of the larvae had cramped their larval arms and spread out the spines of the ER. By the end of the next 15

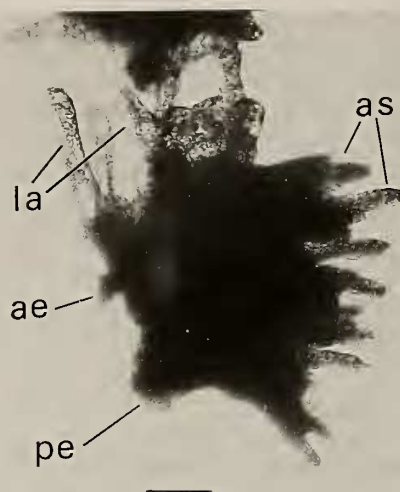


FIG. 7. An ER-exposed larva after treatment with GABA. Competent larvae were treated with 10^{-5} M GABA for 8.5 hr and observed after 48 hr. la, larval arms; ae, anterior epaulet; pe, posterior epaulet; as, adult spines. Spines in ER are exposed but no primary podium is observed. Larval arms remain unretracted. Bar = $100\ \mu\text{m}$.

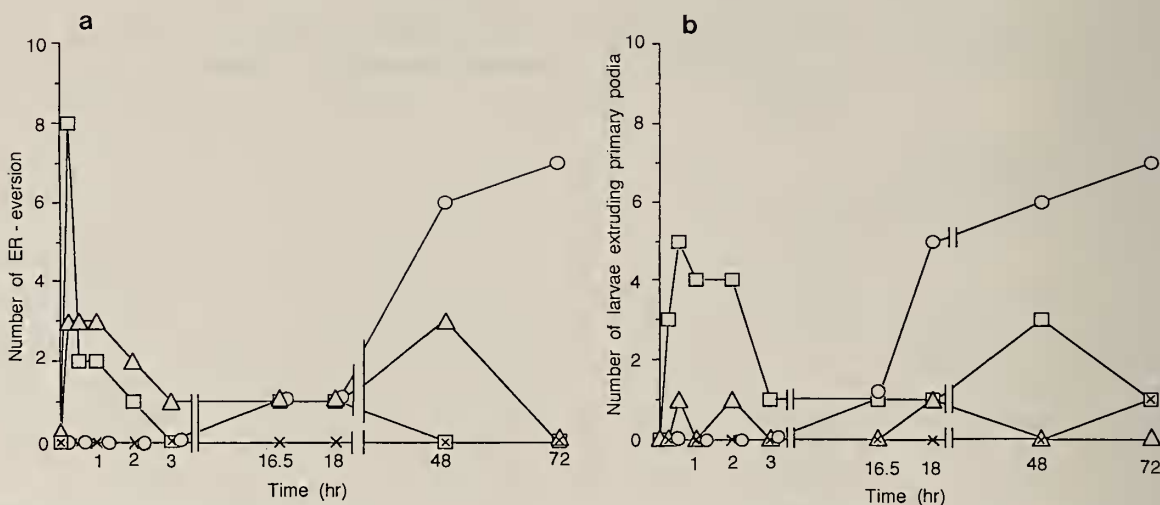


FIG. 8. Effects of GABA on extruding of primary podia and eversion of the echinus rudiment. Each 10-larvae group was treated with 10^{-6} M (Δ), 10^{-7} M GABA ($\square$) and 6×10^{-7} M L-glutamine ($\circ$) for 18 hr. A control group was kept in MFSW alone ($\times$). 15 min from beginning of treatments, GABA-treated larvae everted the echinus rudiments (a) and extruded the primary podia (b), but later returned to their original larval form.

min, however, the number of cramped larvae decreased to two (Fig. 8a). The cramp of larvae occurred instantly after exposure to GABA solution, and then resolved within 1 hour followed by gradual closure of the spine. The primary podia were also retracted later, although initially extended in about half the larvae at the lower concentration (10^{-7} M) of GABA (Fig. 8b). At 10^{-6} M GABA, almost no larvae extended podia (Fig. 8b). The higher concentration of GABA may be toxic. After all, GABA induced rapid changes in the larval arms and spines of ER and did not induce metamorphosis or eversion of the ER, as glutamine did (Figs. 8a, 8b).

DISCUSSION

During the metamorphosis of sea urchin larvae, an eversion of the echinus rudiment (ER), histolysis and resorp-

tion of larval body and growth and differentiation of adult tissues occurred. The time courses of these developmental phenomena differ according to each species.

In *Lytechinus pictus* and *D. excentricus*, the echinus rudiment is everted 3–5 min after receiving natural cues; the bacterial film and the sands in the adult habitat, and the larval body subsequently collapses after an additional 15–30 min [5, 9]. After these initial phases of metamorphosis, the juvenile undergoes a period of extensive reorganization and differentiation. In other sea urchin species, we do not have detailed information about the time process of metamorphosis after exposure to natural cues. Most larvae of six species of sea urchins (*P. depressus* and others) metamorphosed within 3 days after the addition of bacterial films into culture vessels [23].

In the present study, metamorphosis of *P. depressus*

larvae was induced by L-glutamine. The glutamine-induced metamorphosis began with cessation of larval swimming within 1 hour, followed by the retraction of larval arms at 6–9 hr, and finally, eversion of the echinus rudiment at 12–38 hr (Fig. 3). When larvae of *Hemicentrotus pulcherrimus* were treated with L-glutamine, the time processes were similar to *P. depressus* in the present study (Yazaki, unpublished data). The early changes of metamorphosis; the cessation of swimming and the retraction of arms, seems not to be linked to the eversion of the echinus rudiment, because, while a 2-hr treatment with glutamine was enough to induce the cessation of swimming and the retraction of larval arms (Figs. 4a, 4b), a 4- to 8-hr treatment was necessary to induce ER-eversion (Fig. 4d).

Cameron and Hinegardner [4] and Strathman [19] have suggested that the sucker tips of the primary podia are the location of sensory organs that are responsible for the perception of the substratum-associated cues (rocks and shells from adult habitat) to metamorphose. Sensory cells of primary podia in *D. excentricus* were localized to the podial sucker by Burke [1]. On the basis of the experiments using ablation and electrical stimulation, he further proposed that the apical neuropile and the oral ganglion of competent larvae are nerve centers that both mediate between the perception of natural cues and control the initiation of metamorphosis [2].

In the present experiment, the primary podia extruded in most larvae 6–7 hours after the start of glutamine treatment (Fig. 3), with the podial extrusions being independent of the elapsed treatment time with glutamine. Even if the treatment time was 1 hr, the primary podia were extruded, while a 4- to 8-hr treatment with glutamine was necessary for inducing ER-eversion (Fig. 4d). Accordingly, it is apparent that glutamine induced the extrusion of primary podia, and that the podia were always extruded when the ER was everted, but the ER did not necessarily evert in larvae whose podia had extruded.

In the central nervous system of mammals, it is known that glutamine is metabolized to glutamic acid by deamination, and then GABA is formed by decarboxylation of glutamic acid. In *P. depressus*, glutamic acid did not induce metamorphosis, and GABA induced a rapid and transient cramp of larval arms and spread out the spines of adult rudiment, but did not go on to induce eversion of the ER of the larvae.

In abalone larvae, GABA induces settlement and complete metamorphosis, but L-glutamine is absolutely inert [13]. At an optimal GABA concentration (10^{-6} M), settlement is rapidly induced, followed by a loss of the ciliated columnar epithelial cells of the velum within 15–20 hr and formation of a new adult shell ca. 40 hr after the addition of GABA. In the induction of the behavioral and developmental metamorphosis of abalone by GABA, cyclic AMP, calcium, and a glycopeptide secretion from the cephalic sensory complex are thought to mediate transduction of the GABA signal in the control of behavioral and morphogenic changes [14]. GABA at high concentrations over 10^{-5} M does not induce

the developmental metamorphology (new shell synthesis and de-ciliation), although there is a rapid induction of settling [14]. This is similar to the larvae of *P. depressus* which were treated with 10^{-5} M GABA, i. e., the early changes (cessation of swimming and cramp of arms) were induced, but the later changes (extruding of podia and eversion of ER) were not.

In sea urchins, the apical surface of the larval epithelium is distributed by the egg-originated surface substance (ES-1) [21]. During glutamine-induced metamorphosis, ES-1-containing cells were found to be dispersed in larvae whose arms were retracting, and similar ES-1-containing cells were also found among coelomocytes of adult sea urchins [22]. These ES-1-containing cells may be attributable to the resorption of larval tissues by phagocytosis. In addition, it was found in *H. pulcherrimus* that the competent larvae exhibited high levels of mitotic activity in the epithelial cells of the arms and epaulets. When such larvae were treated with glutamine to induce metamorphosis, the high mitotic activity in epithelial cells decreased, while it did not change when the glutamine treatment was insufficient for ER-eversion (Yazaki, unpublished data). Thus glutamine-sensitive changes in mitotic activity may also be included in sea-urchin metamorphosis induced by glutamine.

ACKNOWLEDGMENTS

The authors would like to thank Dr. K. Kuwasawa for his critical reading of the manuscript, and Drs. H. Shirai and T. Yazawa for their valuable discussions and encouragements. We also express thanks to Dr. K. Inaba and Misaki marine Biological Station for providing the sea urchin, *P. depressus*.

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